TECHNICAL MANUSCRIPT 255

FACTORS AFFECTING TRANSFORMATION OF BACILLUS LICHENIFORMIS

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NOVEMBER 1965

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

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FACTORS AFFECTING TRANSFORMATION OF *BACILLUS LICHENIFORMIS*

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Project 1C014501B71A01

November 1965

ABSTRACT

Transformation systems involving two types of transformable mutants of Bacillus licheniformis 9945A were compared. Each system required its specific growth medium but a single transformation medium could be used for both. Cells from a culture of optimum age developed competence during incubation in a completely synthetic transformation medium. With each system 3 to 5% of the recipient cells were transformed upon exposure to wild-type DNA for 2 to 3 hours. When competent cells were exposed to DNA for 30 minutes, 1 to 2% of them were transformed. The data are interpreted to mean that cells were heterogeneous with respect to development of competence, and when properly grown cells were incubated in transformation medium, some of them gained competence while others lost it. If DNA was present during the entire period, the cells were transformed as they became competent and the transformants accumulated. However, during any short period of exposure to DNA only those cells that were competent at the time were potential transformants. The high frequencies of transformation obtained in these studies made it feasible to prepare marked strains by transforming markers into recipient cells. These experiments demonstrated that the characteristics of the two transformation systems could not be attributed to specific nutritional markers. Presumably each of the two series of highly transformable auxotrophic mutants also carried at least one other mutation that resulted in development of competence under the specific conditions.

CONTENTS

TABLES

I. INTRODUCTION

Using a screening procedure based on the occurrence of transformation from auxotrophy to prototrophy on minimal agar plates, Gwinn and Thorne[1] showed that certain mutants derived from Bacillus licheniformis 9945A were transformable when grown under the test conditions employed. One of these mutants, M28 (glycine$^-$), was studied further and some factors affecting its transformation were investigated, although under the best conditions only about 10^{-2}% of the recipient cells transformed. The parent wild-type strain produces large amounts of glutamyl polypeptide when grown appropriately,[2] and on minimal agar plates it produces mucoid colonies (peptide$^+$). The mutant M28 was rough (peptide$^-$) and with appropriate deoxyribonucleic acid (DNA) it could be transformed to peptide$^+$ as well as to prototrophy. The rough character of M28 was related to its ability to be transformed, because glycine$^-$ peptide$^+$ strains prepared by transforming M28 to peptide$^+$ were much less susceptible to transformation to glycine$^+$ than the original M28.

Using the same screening procedure employed by Gwinn and Thorne,[1] Leonard et al.[3] also found certain mutants to be transformable when grown under appropriate conditions. These workers confirmed the findings that the ability to synthesize glutamyl polypeptide is a transformable characteristic and that mucoid auxotrophs (peptide$^+$) transformed much less readily than rough auxotrophs (peptide$^-$). In a more recent report Leonard and Mattheis[4] described improvements in the transformation system they developed and reported the isolation of colonial variants that transformed at higher frequencies than the original isolates.

Our present report gives improved conditions for transformation of the original M28 mutant and describes the isolation, by a procedure different from that described by Leonard and Mattheis,[4] of derivatives of M28 that transform at greater frequencies. Included also are results of studies in which the conditions for transforming the M28 series of mutants, referred to here as the M28 system, were compared with the different conditions that were described for transforming the mutants isolated by Leonard and Mattheis.[4] M18 was chosen as being typical of their mutants and this system will be referred to as the M18 system. The two systems arose as a result of the fact that different mutants require different conditions for growth of cells capable of developing competence. An attempt is made to bring together in one place the essential features and procedures of both systems to facilitate the use of B. licheniformis transformation by other workers who may wish to use it as a tool in genetics studies.

II. MATERIALS AND METHODS

A. ORGANISMS

The wild-type prototrophic strain was B. licheniformis ATCC 9945A. All the mutants were derived from 9945A and they are listed and described in Table 1.

TABLE 1. DESCRIPTION OF B. LICHENIFORMIS 9945A MUTANTS[a/]

Designation	Description, Origin, or Reference
M28	gly⁻, by UV irradiation of 9945A.[1]
M28-RT	Prototrophic transformant of M28.[1]
M28-RT-M20	gly/ser⁻, by UV irradiation of M28-RT (Table 4).
M28-RT-M20-RT	Prototrophic transformant of M28-RT-M20.
M28-RT-M20-RT-T4	his⁻, derived by transforming his⁻ marker of M28-D14-3-RT-M8 into M28-RT-M20-RT
M28-RT-M20-D1 and D5	gly/ser⁻ his⁻, derived by transforming his⁻ marker of M28-D14-3-RT-M8 into M28-RT-M20.
M28-RT-M20-RT-T10	ad⁻, derived by transforming ad⁻ marker of M17 into M28-RT-M20-RT.
M28-D14-3	leu⁻ gly⁺ transformant of M28-D14 (leu⁻ gly⁻) of Gwinn and Thorne.[1]
M28-D14-3-RT	Prototrophic transformant of M28-D14-3.
M28-D14-3-RT-M8	his⁻, by UV irradiation of M28-D14-3-RT.
M17	ad⁻, colonial type II transformable mutant described by Leonard and Mattheis.[4]
M18	arg⁻, colonial type II transformable mutant described by Leonard and Mattheis.[4]
M18-D1	arg⁻ ad⁻, derived by transforming ad⁻ marker of M17 into M18.

a. Abbreviations: gly, glycine; gly/ser, glycine or serine; his, histidine; ad, adenine; leu, leucine; arg, arginine; UV, ultraviolet light; M, mutant; T, transformant; RT, rough prototrophic transformant; D, doubly auxotrophic. All the mutants listed were rough, i.e. peptide⁻.

B. MEDIA

Most of the media used in the transformation experiments are listed in Table 2. Minimal 10 was minimal 1 supplemented with L-alanine, DL-valine, L-leucine, L-isoleucine, L-serine, and L-threonine, each at a concentration of 320 mg/liter. We developed this medium for Bacillus cereus but it was shown to give good recovery of B. licheniformis transformants. Transformants grew faster on it than on minimal 1 and therefore it was often used with auxotrophs not requiring any of the six supplemental amino acids. NBY medium was composed of 8 g of Difco nutrient broth and 3 g of Difco yeast extract per liter. Triple distilled water was used to prepare all media. Solid media were prepared with 15 to 20 g of agar per liter.

In preparation of minimal 1 and minimal 10 agar all the constituents except $FeCl_3$, glucose, and agar were autoclaved together at double strength, and equal volumes of this and sterile double strength molten agar were combined. $FeCl_3$ and glucose were always added aseptically. When it was necessary to add supplements to minimal agar in order to score transformants they were added aseptically in the following amounts (in mg/ml); glycine, 1.0; other amino acids, 0.03; adenine sulfate, 0.03; Difco casamino acids, 1.0 or 0.1 as indicated.

NBSG-X broth was prepared by dissolving the phosphates and $(NH_4)_2SO_4$ in about one-half the final volume of water, adjusting the pH to 8.0 with NaOH, adding this solution to a second solution containing the nutrient broth, $MgSO_4$, and sodium citrate, and adding water to make a final volume of 1 liter. The medium was sterilized in 100-ml portions in 250-ml flasks and just before inoculation the following were added to 100 ml: 0.5 ml of $FeCl_3 \cdot 6H_2O$ (8.0 mg/ml), 0.1 ml of $MnSO_4 \cdot H_2O$ (2.5 mg/ml), and 2.0 ml of glycerol (250 mg/ml). The desired amount was then transferred to a sterile flask.

In preparation of the other media listed in Table 2, NBSG, TM, BLSG, and BL, the following constituents were sterilized individually and added aseptically: $FeCl_3$, $MnSO_4$, $CaCl_2$, NaCl, L-tryptophan, glucose, glycerol, and hydrolyzed casein.

C. CULTURAL CONDITIONS

Spores were produced in potato extract medium as described by Thorne.[5] Cells for transformation were grown in the appropriate broth in Erlenmeyer flasks inoculated with 1×10^7 to 8×10^7 spores per flask and incubated on a reciprocal shaker (5-cm stroke, 100 excursions per min). With BLSG medium the recommended procedure[3,4] of using 50 ml in 500-ml flasks was always followed and with NBSG-X, unless otherwise stated, 25 ml were used in 250-ml flasks. The incubation time for cultures was usually 18 hours but this varied in some experiments and the exact time is always given with the results. Viable cell counts were done by diluting cells in peptone (1% w/v) and spreading 0.1 ml on NBY agar plates. All incubations were at 37 C.

TABLE 2. MEDIA USED IN TRANSFORMATION EXPERIMENTS[a/]

Constituent	Minimal 1[8]	NBSG[1]	NBSG-X	TM	BLSG[3,4,b/]	BL[3,5,b/]
K_2HPO_4	14.0	14.0	56.0	14.0	14.65	14.65
KH_2PO_4	6.0	6.0	24.0	6.0	5.5	5.5
$(NH_4)2SO_4$	2.0	2.0	2.0	2.0	2.0	2.0
Sodium citrate·$2H_2O$	1.0	1.0	6.0	1.0	3.0	3.0
$MgSO_4 \cdot 7H_2O$	0.20	0.20	1.2	1.0	0.2	1.43
$FeCl_3 \cdot 6H_2O$	0.04	0.04	0.04		0.16	
$MnSO_4 \cdot H_2O$	0.00025	0.00025	0.0025	0.0125	0.002	0.150
$CaCl_2 \cdot 2H_2O$				0.30	0.15	0.150
NaCl				11.7[d/]		
L-Tryptophan						0.005
Glucose	5.0			5.0		5.0
Glycerol		5.0	5.0		12.0	
Difco nutrient broth		8.0	8.0		8.0	
L-Glutamic acid	2.0					
Hydrolyzed casein[c/]						0.10
pH	7.0	7.0	8.0	7.0	7.0	7.0

a. Amounts of constituents are given in grams per liter.
b. C.G. Leonard, personal communication.
c. Vitamin-free acid-hydrolyzed casein (Nutritional Biochemicals Corp., Cleveland, Ohio).
d. This amount of NaCl (0.2 M) does not include that added with the DNA to transformation mixtures.

D. TRANSFORMATION

All transformations were carried out in a final volume of 1 ml in test tubes (18 x 150 mm) incubated in a slanted position on the shaker (described above) at 37 C. After the desired incubation time, 0.05 ml (50 μg) of deoxyribonuclease (once crystallized, Worthington Biochemical Corp., Freehold, N.J.) was added and incubation was continued for 15 minutes. In experiments with the M28 series of mutants one of the following two procedures was used: (i) one-tenth ml of culture was added to 0.8 ml of TM broth in a tube and 0.1 ml of DNA was added as desired, either at the beginning of the experiment or after suitable periods of incubation. (ii) Cultures were diluted 1:10 or more in TM broth and 25 ml were incubated in a 250-ml shaken flask. At the desired times 0.9-ml samples were removed to tubes with 0.1 ml of DNA. In experiments with M18 and M18-D1, cultures were diluted about 1:20 in EL broth; 25 ml in 250-ml flasks or 0.9-ml samples in tubes were incubated with shaking. After the desired periods of time 0.1 ml of DNA was added to 0.9 ml of cells. Since details of the transformation procedure varied somewhat from one experiment to another the exact procedure is always given with the results. In all the experiments reported here the final concentration of DNA in transformation mixtures was 32 μg/ml; this amount was shown to be en excess.

For scoring transformants samples were diluted in the same kind of broth used in the transformation mixtures and plated on the appropriate minimal agar. Transformants of M28 were conveniently scored on minimal agar with 0.1% of casamino acids. This amount of casamino acids did not contain enough glycine to allow the glycine auxotroph to grow into full-sized colonies, but it allowed the transformants to grow faster. With the gly/ser$^-$ mutant a concentration of casamino acids greater than 0.01% gave too much background growth of the auxotrophic cells. Transformants of the M18 series of mutants could be scored after plates were incubated for 36 to 40 hours. The M28 series of mutants grew more slowly and transformants could not be scored accurately before 48 hours. All the mutants used in these experiments were very stable; revertants were rarely, if ever, seen. However, appropriate controls for detecting revertants were always included in transformation experiments.

E. DNA

DNA was prepared as described by Gwinn and Thorne.[1] Cells for DNA extraction were grown in NBY broth as described by those authors except that the inoculum of spores was reduced to 1×10^3 per ml. With this amount of inoculum very few, if any, spores were present in 16-hour cultures and there was no difficulty in preparing sterile DNA. For storage at 5 C as well as for use in experiments, DNA was in solution in 2 M NaCl. DNA was determined by the method of Burton.[8]

III. RESULTS

A. TRANSFORMATION FREQUENCIES OF M28 CELLS GROWN UNDER VARIOUS CONDITIONS

NBSG medium was the best of the media tested by Gwinn and Thorne[1] for growing recipient cells of M28 for transformation, although only about 10^{-2}% of the cells transformed under the test conditions. The present studies began with attempts to improve the frequencies of transformation with M28, and these experiments resulted in the modification of NBSG. The new medium, NBSG-X, is the same as NBSG except that the concentrations of phosphates were increased fourfold, the concentrations of $MgSO_4$ and citrate were increased sixfold, and the concentration of $MnSO_4$ was increased tenfold. Of all these modifications the most important one was the increase in phosphate to the unusually high concentration of approximately 0.5 M. While these tests on growth medium were being done, experiments were also carried out to improve the medium used for the transformation process. In the original studies Gwinn and Thorne[1] used minimal 1 broth, but after many tests, we adopted TM broth, pH 7.0, as the routine transformation medium. This is the same as minimal 1 broth except that glutamic acid was omitted because it inhibited transformation, the $MgSO_4$ concentration was increased fivefold, the $MnSO_4$ concentration was increased 50-fold, and $CaCl_2$ and NaCl were added. In numerous tests in which each of the constituents was tested over a range of concentrations and in which media at various pH values were tested, the best results were obtained with the medium as described. The optimum NaCl concentration was 0.3 to 0.4 M. The routine addition of 0.1 ml of DNA in 2 M NaCl to 0.9 ml of cells in TM containing 0.2 M NaCl resulted in a final molarity in the optimum range.

Table 3 shows some transformation frequencies obtained with cells grown in NBSG-X medium and transformed in TM broth. These data demonstrate the effects of pH of the growth medium and degree of aeration as controlled by the volume of medium in the 250-ml flasks. Two combinations of volume and pH, 50 ml at pH 6.0 and 25 ml at pH 8.0, gave maximum transformation frequencies. With 50 ml of medium at pH 6.0 the optimum time of incubation was 46 to 54 hours even though the viable cell count reached the maximum (about 2×10^9/ml) after 10 to 12 hours. Little or no change in numbers of viable cells could be detected throughout the remainder of the period and very little change in pH, less than 0.2 unit, occurred during the entire time.

Although the highest frequencies were obtained with cells grown at pH 6 in a volume of 50 ml, the long incubation period required was inconvenient. Therefore, the next best set of conditions, 25-ml volume and pH 8.0, was adopted for further studies. When M28 was grown for 18 to 20 hours under these conditions, 0.1 to 0.3% of recipient cells were transformed when incubated with DNA in TM broth for 2 to 3 hours. For the remainder of the experiments reported here, except when otherwise stated, cells of the M28 series of mutants were grown in this manner.

TABLE 3. TRANSFORMATION OF M28 (gly⁻) CELLS GROWN UNDER VARIOUS CONDITIONS[a]

Medium		Optimum Incubation Time, hours	Transformation to Prototrophy, %
pH	ml/250-ml Flask		
6.0	50	46 to 54	0.1 to 0.6
7.0	50	24 to 26	0.02 to 0.04
8.0	50	24 to 26	0.03 to 0.05
7.0	25	18 to 20	0.03 to 0.1
8.0	25	18 to 20	0.1 to 0.3

a. The medium was NBSG-X. One-tenth ml of cells, 0.1 ml of wild-type DNA, and 0.8 ml of TM broth were incubated for 3 hr. Transformants were scored on minimal 1 agar supplemented with 0.1% of casamino acids.

Early tests revealed that these conditions, which were optimum for M28, were not specific for cells with the gly⁻ marker. M28-D14-3 is a gly⁺ leu⁻ transformant derived from M28-D14 (gly⁻ leu⁻), by Gwinn and Thorne.[1] When it was tested by the standard procedure adopted for M28, 0.6 to 0.7% of the cells were transformed during a 3-hour exposure to DNA.

B. TRANSFORMATION FREQUENCIES OF MUTANTS DERIVED FROM M28

Spores of a rough, prototrophic transformant of M28, M28-RT, were irradiated with UV to induce mutations and new auxotrophic mutants were isolated. Fifty of these were tested for ability to be transformed and some of the results are shown in Table 4. The transformation frequencies varied over a wide range from a low of 0.0002% to a high of 3.3%. There was no apparent correlation between nutritional requirements and transformation frequency. M28-RT-M20, which gave the highest frequency, required either glycine or serine for growth. That this was a different marker from the glycine marker in M28 was confirmed by the fact that DNA from one mutant would transform the other to the same extent as wild-type DNA.

Similar results to those above were obtained when new auxotrophic mutants were isolated following UV irradiation of a prototrophic transformant derived from M28-D14-3. Two among the 35 tested gave transformation frequencies higher than the parent; M28-D14-3-RT-M8 (his⁻) and M43 (arg⁻) gave frequencies of 2% and 1.5%, respectively. In these tests the

parent, M28-D14-3, gave a frequency of 0.6%. It is interesting that a mutant requiring either glycine or serine was also isolated in these experiments; however, it transformed at the same frequency, 0.6%, as the parent, M28-D14-3.

TABLE 4. TRANSFORMATION OF MUTANTS DERIVED FROM M28-RT[a/]

Mutant	Requirement	Transformation to Prototrophy, %
M28-RT-M1	methionine	0.7
M76	methionine	0.07
M73	methionine	0.09
M4	leucine	0.6
M20	glycine or serine	3.3
M32	arginine	0.8
M45	purine	0.4
M48	lysine	0.3
M66	histidine	0.03
M56	histidine	0.3
M19	tryptophan	0.002
M40	guanine	0.0002
M85	valine	0.2
M90	isoleucine	0.1
M74	glycine	0.05
M63	glycine	0.7
M28 (control)	glycine	0.1
M28-D14-3 (control)	leucine	0.7

a. Cultures were grown in NBSG-X for 18 hr and 0.1 ml of each was incubated with 0.1 ml of wild-type DNA and 0.8 ml of TM broth for 3 hr. Transformants were scored on minimal 1 agar.

C. TRANSFORMABILITY OF STRAINS PREPARED BY TRANSFORMING MARKERS INTO RECIPIENT CELLS

Since it appeared that changes in capacity to be transformed often occurred during the process of preparing marked strains by irradiating spores with UV and selecting auxotrophic mutants, it seemed desirable to be able to prepare suitably marked strains by transforming markers into highly transformable mutants. Experiments were done to determine whether prototrophic transformants retained the ability to serve as recipient cells for transformation. Two prototrophic transformants (peptide$^-$) resulting from exposing M28-RT-M20 (gly/ser$^-$) to wild-type DNA were selected and

purified by streaking on minimal agar and picking single colonies. Both of these prototrophic transformants retained the ability to develop competence as determined by their capacity to be transformed from *peptide*$^-$ to *peptide*$^+$. An example of the results obtained with one of them, M28-RT-M20-RT, is shown in Table 5.

TABLE 5. TRANSFORMATION OF A *PEPTIDE*$^-$ PROTOTROPH TO *PEPTIDE*$^+$ AND OF STRAINS PREPARED BY TRANSFORMING MARKERS INTO RECIPIENT CELLS[a/]

Recipient Cells			Transformants		
Strain Designation[b/]	Genotype	No./ml	Genotype	No./ml	Transformation, %
M28-RT-M20-RT	*peptide*$^-$, prototroph	3.0×10^8	*peptide*$^+$	1.2×10^7	4.0
M28-RT-M20-RT-T4	*his*$^-$	2.7×10^8	*his*$^+$	1.0×10^7	3.7
M28-RT-M20-D5	*gly/ser*$^-$ *his*$^-$	3.5×10^8	*gly/ser*$^+$ *his*$^+$	1.3×10^7 1.2×10^7	3.7 3.4

a. Recipient cells were grown for 18 hr in NBSG-X and 0.1 ml samples containing the indicated number of cells were incubated for 3 hr with 0.1 ml of wild-type DNA and 0.8 ml of TM broth. Transformants were scored on minimal 1 agar for *peptide*$^+$, on minimal 10 agar for *his*$^+$, and on minimal 1 agar supplemented with histidine and 0.01% of casamino acids for *gly/ser*$^+$.
b. See Table 1 for derivation of the strains.

Competent cells of the prototrophic transformant, M28-RT-M20-RT of Table 5, were transformed with DNA prepared from M28-D14-3-RT-M8 (*his*$^-$) and transformation mixtures were plated on NBY agar to produce isolated colonies. Colonies were picked at random and tested for the ability to grow on minimal 1 agar, and those that did not grow were shown to be *his*$^-$. Six such strains were tested for transformation and each of them transformed to prototrophy at a frequency similar to that obtained with the original recipient parent strain M28-RT-M20. Table 5 includes an example of the results with one of these strains, M28-RT-M20-RT-T4.

In a manner similar to that just described the *his*$^-$ marker of M28-D14-3-RT-M8 was transformed into cells of M28-RT-M20 (*gly/ser*$^-$) and doubly marked strains were isolated. Four of these were tested for transformation and they all transformed well for each of the two markers. Results with one of them, M28-RT-M20-D5, are included also in Table 5.

D. SPECIFICITIES OF GROWTH MEDIA FOR THE TWO TRANSFORMATION SYSTEMS

Following the procedure reported by Leonard and Mattheis[4] and using one of their mutants, M18 (arg⁻), we were able to reproduce the frequencies of transformation they reported. We were also able to prepare suitably marked strains in that system by transforming desired markers into appropriate cells. For example, Table 6 gives results of a transformation experiment with the doubly marked strain, M18-D1 (arg⁻ ad⁻). This strain was prepared by transforming M18 (arg⁻) with DNA isolated from M17 (ad⁻). The cells transformed for each of the two markers at a high frequency.

TABLE 6. TRANSFORMATION OF M18-D1 (ARG⁻ AD⁻)[a/]

Recipient Cells/ml	DNA Source	Transformation			
		arg⁺		ad⁺	
		Transformants/ml	%	Transformants/ml	%
8.7×10^7	M18 (arg⁻)	0	0	3.2×10^6	3.7
8.7×10^7	M17 (ad⁻)	4.4×10^6	5.1	0	0
8.7×10^7	9945A	4.8×10^6	5.5	3.4×10^6	3.9

a. Strain M18-D1 (arg⁻ ad⁻) was obtained by transforming M18 (arg⁻) with DNA prepared from M17 (ad⁻). Recipient cells were grown in BLSG for 18 hr. They were diluted 1:20 in BL broth and 0.9-ml samples (8.7×10^7 cells) were incubated in tubes for 2 hr. DNA (0.1 ml) was added and incubation was continued for 2 hr. Transformants were scored on appropriately supplemented minimal 10 agar.

The data in Table 7 demonstrate the specificities of the two growth media, BLSG and NBSG-X, for the M18 and M28 types of mutants, respectively. The results of experiments 1 and 3 in the table show that the two transformation media, TM and BL, gave similar results and that they could be used interchangeably for both systems. The data of experiment 2 show the superiority of BLSG over NBSG-X medium for growing recipient cells of M18-D1. The optimum concentrations of glycerol for the two media are different, 1.2% for BLSG and 0.5% for NBSG-X. However, increasing the concentration of glycerol to 1.2% in NBSG-X did not improve this medium for M18-D1. The results of experiment 3 in Table 7 show that BLSG is inferior to NBSG-X as a growth medium for recipient cells of M28-RT-M20-RT-T10. This was confirmed for other mutants of the M28 series. Reducing the concentration of glycerol to 0.5% in BLSG (experiment 4) did not alter the results significantly. It should be pointed out that the numbers of

cells obtained with any particular mutant in the two media were about the same. It is in the quality of the cells with respect to their transformability that a great difference was observed.

TABLE 7. SPECIFICITY OF GROWTH MEDIA FOR RECIPIENT CELLS OF M18 AND M28 TYPES OF MUTANTS[a]

Exp. No.	Mutant	Growth Medium	Transformation Medium	Recipient Cells/ml	Transformation Transformants/ml	%
1	M18-D1 (arg⁻ ad⁻)	BLSG	BL	1.4×10^8	1.4×10^6 (arg⁺)	1.0
			TM	1.1×10^8	1.6×10^6 (arg⁺)	1.5
2	M18-D1 (arg⁻ ad⁻)	BLSG	TM	6.2×10^7	7.3×10^5 (ad⁺)	1.2
		NBSG-X	TM	1.2×10^8	9.0×10^3 (ad⁺)	0.008
		NBSG-X[b]	TM	7.8×10^7	$<1 \times 10^3$ (ad⁺)	<0.001
3	M28-RT-M20-RT-T10 (ad⁻)	BLSG	BL	1.7×10^8	9.5×10^4 (ad⁺)	0.06
		NBSG-X	TM	2.2×10^8	1.7×10^6 (ad⁺)	0.8
		NBSG-X	BL	2.3×10^8	1.6×10^6 (ad⁺)	0.7
4	M28-RT-M20-RT-T10 (ad⁻)	BLSG	TM	1.1×10^8	3.6×10^4 (ad⁺)	0.03
		BLSG[c]	TM	1.4×10^8	6.2×10^4 (ad⁺)	0.04
5	M28-RT-M20-RT-T10 (ad⁻)	NBSG-X	TM	2.7×10^8	1.2×10^7 (ad⁺)	4.4

a. Recipient cells of M18-D1 were grown for 18 hr and those of M28-RT-M20-RT-T10 for 16 hr, the respective optimum times. Except for experiment 5, cells were diluted 1:20 in transformation medium and 25 ml were incubated in 250-ml flasks for 4 hr. Samples (0.9 ml) were then incubated with 0.1 ml of wild-type DNA for 30 min. In experiment 5, 0.1 ml of cells, 0.1 ml of DNA, and 0.8 ml of TM broth were incubated together for 3 hr. Transformants were scored on minimal 10 agar supplemented as necessary.

b. The concentration of glycerol was increased from 0.5% to 1.2%, the optimum concentration for M18-D1 in BLSG.

c. The concentration of glycerol was reduced from 1.2% to 0.5%, the optimum concentration for the M28 series of mutants in NBSG-X.

Routinely BLSG was used in 50-ml amounts in 500-ml flasks, since this was the recommended procedure.[4] However, in experiments not shown here, results with M18-D1 grown in 25 ml of BLSG in 250-ml flasks were similar to those obtained routinely.

The difference between the M28 and M18 types of mutants does not appear to be attributable to the presence of particular nutritional markers. The data in Table 7 provide evidence for this. The ad⁻ marker was transformed into each of the two mutants used in these experiments with the use of DNA isolated from M17 (ad⁻). M17 is highly transformable under the same conditions that are optimum for M18.[4] When the ad⁻ marker of M17 was put into M18 (arg⁻) the characteristics of this mutant with respect to transformation did not change. Likewise, the original specificity of M28-RT-M20 with respect to growth medium for optimum transformation was retained when the ad⁻ marker of M17 was put into M28-RT-M20-RT.

E. DEVELOPMENT OF COMPETENCE IN TRANSFORMATION MEDIUM

We, as well as Leonard and Mattheis,[4] had assumed that cells of transformable mutants of B. licheniformis became competent in their growth medium late in the stationary phase. The fact that the age of the culture was a critical factor in controlling the frequency of transformation contributed to this belief. However, in experiments designed to test this we obtained evidence that cells did not become competent, at least to any great extent, in their growth medium, but that appropriately grown cells developed competence during incubation in transformation medium. The data in Table 8 demonstrate that cells of M28-RT-M20-D1 (gly/ser⁻ his⁻) became competent during incubation in TM broth. In this experiment samples of cells in TM broth were incubated in a series of tubes and at hourly intervals DNA was added to one of the tubes and incubation was continued for 30 minutes. Without any period of incubation in TM broth before addition of DNA, only 0.1% of the cells transformed; with longer periods of incubation in TM broth the proportion of transformed cells increased to a maximum of about 2%. In several experiments maximum competence was achieved after cells were incubated in TM broth for about 3 to 4 hours and this high level of competence was maintained through the 6th hour. After that time the number of transformants decreased but even after 8 hours, which was the longest period tested, about 0.5% of the cells transformed. Control experiments were done in which cells were allowed to continue incubating in their growth medium. At hourly intervals 0.1-ml samples were removed and incubated for 30 minutes with 0.1 ml of DNA and 0.8 ml of TM. In such tests the frequencies of transformation were always several-fold lower than those obtained with cells of the same culture incubated for an optimum period in transformation medium.

TABLE 8. DEMONSTRATION OF DEVELOPMENT OF COMPETENCE IN TM BROTH[a/]

Incubation Time in TM Broth Before Addition of DNA	Incubation Time with DNA	Transformation			
		gly/ser$^+$		*his*$^+$	
		Transformants/ml	%	Transformants/ml	%
hr	min				
0	30	3.0×10^6	0.12	2.0×10^6	0.03
1	30	3.1×10^6	1.3	1.6×10^6	0.67
2	30	3.7×10^6	1.5	2.4×10^6	1.0
3	30	5.2×10^6	2.2	3.0×10^6	1.2
4	30	4.2×10^6	1.8	3.5×10^6	1.5
5	30	4.8×10^6	2.0	4.6×10^6	1.9
0	180	1.3×10^7	5.4	1.1×10^7	4.6

a. Recipient cells of strain M28-RT-M20-D1 (*gly/ser*$^-$ *his*$^-$) were grown for 18 hr in NBSG-X. Tubes concaining 0.8 ml of TM broth and 0.1 ml of cells (2.4×10^8) were incubated for the times indicated. Wild-type DNA (0.1 ml) was added, and incubation was continued for the times indicated. Transformants were scored on minimal 10 agar for *his*$^+$ and minimal 1 agar supplemented with histidine and 0.01% of casamino acids for *gly/ser*$^+$.

Table 9 demonstrates that M18-D1 also developed competence in transformation medium. Results were similar to those obtained with the M28 series of mutants. With both types of mutants determinations of viable cells at periods up through 6 hours indicated that the number of cells did not change significantly during incubation in transformation medium. Also, with each of the two types of mutants, cells developed competence in transformation medium equally well whether 25-ml samples were incubated with shaking in 250-ml flasks or 0.9-ml samples were incubated with shaking in test tubes.

F. EFFECT OF TIME OF EXPOSURE TO DNA

Table 10 shows the effect of various periods of exposure to DNA on the number of transformants obtained. With cells that had been incubated for an optimum time in transformation medium, greater than 0.1% transformation was obtained during an exposure period of 5 minutes. Between 1% and 2% of the cells were transformed in 15 minutes and the number of transformants increased still further in 30 minutes.

TABLE 9. ATTAINMENT OF COMPETENCE DURING INCUBATION OF M18-D1 (*arg*⁻ *ad*⁻) CELLS IN BL BROTH[a]

Incubation Time in BL Broth Before Addition of DNA	Incubation Time with DNA	Transformation to *ad*⁺	
		Transformants/ml	%
hr	min		
0	30	8.0×10^3	0.006
1	30	9.0×10^4	0.07
2	30	6.7×10^5	0.52
3	30	1.7×10^6	1.3
4	30	2.0×10^6	1.5
5	30	2.0×10^6	1.5
6	30	2.0×10^6	1.5
2	120	5×10^6	3.8

a. Cells grown for 18 hr in BLSG were diluted 1:20 in BL broth and 0.9-ml samples (1.3×10^8 cells) were incubated in tubes. After the designated times 0.1 ml of DNA was added and incubation was continued for the times indicated. A total cell count on the 6-hr tube gave 1.2×10^8 cells per ml, indicating that the number of cells did not change significantly during the course of the experiment. Transformants were scored on minimal 10 agar supplemented with arginine.

TABLE 10. TRANSFORMATION FREQUENCIES WITH CELLS OF M18-D1 EXPOSED TO DNA FOR VARIOUS PERIODS OF TIME[a]

Minutes of Exposure to DNA	Transformation			
	ad⁺		*arg*⁺	
	Transformants/ml	%	Transformants/ml	%
1	2.0×10^4	0.00003	1.8×10^5	0.0003
5	9.2×10^4	0.14	1.1×10^5	0.17
15	1.0×10^6	1.6	1.1×10^6	1.7
30	1.6×10^6	2.5	2.2×10^6	3.4
120	5.1×10^6	7.9	5.6×10^6	8.7

a. Cells were grown for 18 hr in BLSG, diluted 1:16 in BL broth, and incubated 5 hr (25 ml/250-ml flask). Samples (0.9 ml) containing 6.4×10^7 cells were incubated with 0.1 ml of wild-type DNA for the indicated times. For the sample exposed to DNA for 120 min, 0.9 ml of cells was removed from the flask after 2 hr and incubated with 0.1 ml of DNA for 2 hr. Transformants were scored on minimal 10 agar supplemented with arginine for *ad*⁺ and with adenine for *arg*⁺.

An interesting fact is that with both types of mutants, exposure to DNA for periods longer than 30 minutes always produced more transformants (Tables 8, 9, and 10). The maximum number of transformants was obtained when cells were exposed to DNA for 2 to 3 hours.

IV. DISCUSSION

These experiments demonstrate that two types of mutants derived from B. licheniformis 9945A had different requirements with respect to optimum medium for growth of transformable cells. The original medium used for both types of mutants was NBSG (Table 2) and it was later modified into BLSG for the M18 type of mutants[4] and NBSG-X for the M28 type. The most important difference in the two modified media is in the phosphate concentration. Apparently the physiology of M28 type is such that the high phosphate concentration, almost 0.5 M, is conducive to the development of transformable cells. Glycerol is an important constituent in the medium for both mutant types, although the optimum concentrations vary somewhat. When glucose was substituted for glycerol, very few if any transformants were obtained. Glycerol inhibits sporulation of B. licheniformis[1] and we believe this is one reason it is such a critical factor in the medium for growing transformable cells.

Except for their specificities with respect to medium for growth of transformable cells, the two types of mutants were very much alike. Each became competent in either of the two transformation media studied; the two had similar colonial morphologies; both were phenotypically as well as genotypically rough (peptide$^-$); and each type included variants that gave a wide range of transformation frequencies.

Information given in this report apparently eliminates specific auxotrophic markers as being responsible for the peculiarities of the two mutant types. When the ad$^-$ marker from a representative of one type was transformed into a representative of the second type, the specificity of the second type with respect to growth medium was not changed. Our hypothesis to account for the two types is that coincidentally to the original mutation resulting in auxotrophy, a second mutation occurred resulting in a physiological change that rendered the cells transformable when grown under certain conditions. According to this hypothesis this coincidental mutation would be different in the two mutant types and one should be able to transform one type to the other. We have not tried to do this.

Although, according to transformation tests, competence developed during incubation of cells in transformation medium and persisted for several hours, the number of transformants that could be obtained in any 30-minute period of exposure to DNA during the hours of maximum competence was never as high as the number that could be obtained by using longer

periods of exposure. This is interpreted to mean that populations of cells were heterogeneous with respect to development of competence and that during incubation in transformation medium some cells gained competence while others lost it. When DNA was present for a long period of time, cells were transformed as they became competent and transformants accumulated throughout the period. However, when exposure to DNA was held to a shorter period, only those cells that were competent at the time were transformed. Alternative possibilities are that individual cells gained, lost, and regained competence in cycles, or that some cells took up DNA much more slowly than other cells. An obvious possibility is that during longer periods of exposure to DNA the number of transformants increased as a result of cell division (growth) of the transformants; this was ruled out in experiments in which continued incubation of transformation mixtures for several hours after deoxyribonuclease treatment did not result in increased numbers of transformants.

Although populations of cells developed maximum competence only after being transferred from growth medium to transformation medium, the length of incubation time in growth medium was a critical factor. Apparently in growth medium cells developed a condition that rendered them potentially competent. Although cultures exhibited this condition to some extent for several hours, the maximum potential for competence was usually not maintained for more than 1 to 3 hours. Neither the nature of the condition that renders the cells potentially competent nor the nature of the condition of competence itself is understood. The situation is somewhat, but not completely, analogous to that in the Bacillus subtilis transformation system.[7] Cultures of B. subtilis strain 168 reached the condition of maximum potential competence during the late logarithmic growth period; cultures of transformable mutants of B. licheniformis did not reach this stage until several hours after onset of the stationary phase. When a potentially competent culture of B. subtilis was diluted 1:10 in a second growth medium, cells achieved competence toward the end of the logarithmic growth period; cells of potentially competent cultures of B. licheniformis developed competence during incubation in a completely synthetic transformation medium that did not support growth of the auxotrophic cells.

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Unclassified
Security Classification

DOCUMENT CONTROL DATA - R&D
(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)
U.S. Army Biological Laboratories
Fort Detrick, Frederick, Maryland, 21701

2a. REPORT SECURITY CLASSIFICATION
Unclassified

2b. GROUP

3. REPORT TITLE
FACTORS AFFECTING TRANSFORMATION OF BACILLUS LICHENIFORMIS

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (Last name, first name, initial)
Thorne, Curtis B.
Stull, Harold B.

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
November 1965	24	7

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO.	Technical Manuscript 255
c. 1C014501B71A01	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

10. AVAILABILITY/LIMITATION NOTICES
Qualified requestors may obtain copies of this publication from DDC.
Foreign announcement and dissemination of this publication by DDC is not authorized.
Release or announcement to the public is not authorized.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	U.S. Army Biological Laboratories Fort Detrick, Frederick, Maryland, 21701

13. ABSTRACT

Transformation systems involving two types of transformable mutants of Bacillus licheniformis 9945A were compared. Each system required its specific growth medium but a single transformation medium could be used for both. Cells from a culture of optimum age developed competence during incubation in a completely synthetic transformation medium. With each system 3 to 5% of the recipient cells were transformed upon exposure to wild-type DNA for 2 to 3 hours. When competent cells were exposed to DNA for 30 minutes, 1 to 2% of them were transformed. The data are interpreted to mean that cells were heterogeneous with respect to development of competence, and when properly grown cells were incubated in transformation medium, some of them gained competence while others lost it. If DNA was present during the entire period, the cells were transformed as they became competent and the transformants accumulated. However, during any short period of exposure to DNA only those cells that were competent at the time were potential transformants. The high frequencies of transformation obtained in these studies made it feasible to prepare marked strains by transforming markers into recipient cells. These experiments demonstrated that the characteristics of the two transformation systems could not be attributed to specific nutritional markers. Presumably each of the two series of highly transformable auxotrophic mutants also carried at least one other mutation that resulted in development of competence under the specific conditions.

DD FORM 1 JAN 64 1473

Unclassified
Security Classification